Case 900-9181/CONT

IN THE UNITED STATES PATENT OFFICE

In re application of:

Daniel Berney

Serial No. 001,479

Filed: January 8, 1979

For: Improvements in or relating to organic compounds

Declaration Under Rule 132

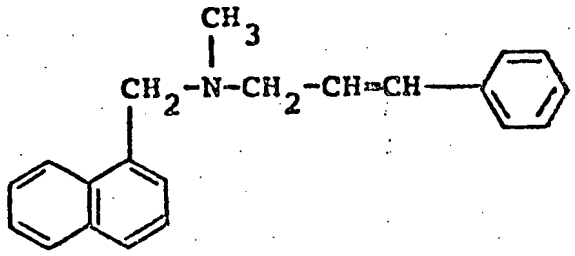
I, Gábor Petrányi, declare and say that:

I am a German citizen residing at Bräuhausgasse 13, A-2320 Schwechat/Austria.

I obtained my doctorate of Veterinary Medicine from Justus Liebig-Universität, Giessen/Germany.

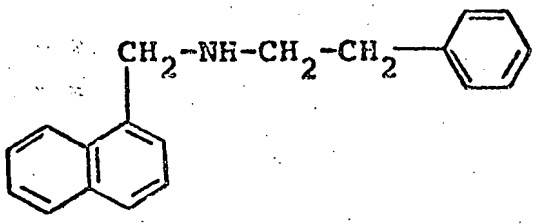
In 1969 I joined the Sandoz Research Institute, Vienna, where I am employed as a mycologist.

Under my supervision, the compound of Example 1 of United States application Serial Number 001,479, namely Trans-N-cinnamyl-N-methyl-N-(1-naphthylmethyl)amine of formula



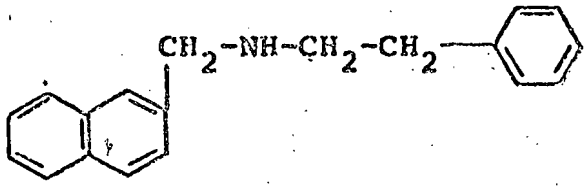
in hydrochloride salt form, hereinafter referred to as Compound A,

N-(1-naphthylmethyl)-N-(2-phenethyl)amine of formula



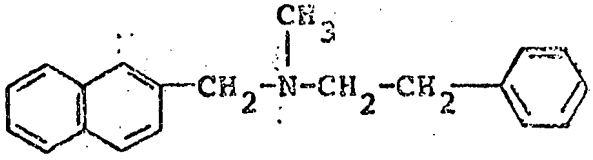
hereinafter referred to as Compound B,

N-(2-naphthylmethyl)-N-(2-phenethyl)amine of formula



hereinafter referred to as Compound C,

and N-methyl-N-(2-napthtylmethyl)-N-(2-phenethyl)amino of formula



hereinafter referred to as Compound D,

were tested in vitro to determine their relative antimycotic activity against various strains of dermatophytes and other fungal strains.

The testing procedures followed and results obtained are described hereinafter.

1. In Vitro Test

1.1 Test Method - Determination of minimum inhibition concentration (MIC) in Microtitre system

The activities of compounds A to D were determined by measuring their minimum inhibition concentration using the series dilution technique with a dilution factor of 2. This was effected on Autotray® - Microtitre plates [rectangular plastic plates with 120 round-bottomed depressions, arranged in 8 rows (coded A to H) each with 15 depressions (coded 1 to 15)] in a Canalco-Autotitre III apparatus [an automatic dilution apparatus with a microdilution head volume of 50 µl].

100 µl of substance solution of known concentration was manually pipetted into the first depression of 8 consecutive rows (A to H). 50 µl of sterile nutrient medium was introduced manually into the remaining depressions.

The 8 geometric dilution series were produced up to the 14th depression of each row, automatically, using the Canalco-Autotitre III apparatus. The 15th depressions served as strain growth controls.

A plate of 50 µl of inoculum - a dilution of a standardised full culture - was manually pipetted into the 120 depressions and the plates, sealed with Parafilm, were incubated for 7 days (dermatophytes), 72 hours (fungi) or 48 hours (yeast) at 30°C and 60 % relative humidity.

The minimum inhibition concentration (MIC) was taken to be the lowest concentration of test substance in the nutrient medium (µg/ml) which completely suppressed strain growth therein to the naked eye.

1.2 Materials

Nutrient Medium: a) Sabouraud Glucose 2% broth (Merck):

Pepton from meat	5 g
Pepton from casein	5 g
D(+)-glucose	20 g

pH = 5.5

b) Sabouraud Glucose 4 % agar (BBL):

Polypepton	10 g
D(+)-glucose	40 g
Agar - agar	15 g

pH = 5,6

c) Fungus agar according to Kimmig (Merck):

Standard II - nutrient broth	15 g
Pepton from meat	5 g
D(+)-glucose	10 g
Sodiumchlorid	5 g
Agar - agar	15 g

pH = 6,5 ± 0,2

Test substance solvent: 5 % dimethyl sulphoxide.

Inoculum: a) dermatophytes:

a number of fungus agar plates according to Kimmig (Merck) were inoculated, incubated for 7 days at 30°C and then carefully, under sterile conditions, scratched with a platinum spatula and, with the help of a glass homogeniser, finely homogenised in Sabouraud glucose 2% broth. The resulting full culture was filled in 1.5 ml portions into plastic ampoules and stored under liquid nitrogen using 5% (v/v) dimethyl-sulphoxide (Merck) as antifreeze agent.

b) fungi: this was carried out in the same manner as for dermatophytes except that sabouraud glucose 4 % agar plates (BBL) were employed and were incubated for 4 days at 30°C.

c) yeasts: this was carried out in the same manner as for dermatophytes except that sabouraud glucose 2 % broth was inoculated with yeast colonies and enriched as a shaken culture for 30 hours at 30° C and 60 % relative humidity.

As control of possible impurities in the full culture maintained under liquid nitrogen, each of 3 fungus agar plates according to Kimmig were inoculated and their germ count per ml determined (10^7 - 10^8/ml).

For the inoculum, the full culture was, before use, immersed in a water bath at 37°C for 2 to 5 minutes and adjusted to the desired germ count (10^3/ml) with Sabouraud glucose 2% broth.

1.3 Test Strains

1.	T. rubrum	Hygiene Inst. Würzburg Prof. H. Seeliger, No. 36
2.	T. rubrum	Dermatologie Würzburg Dr. Barfuss, No. 1895
3.	T. mentagrophytes	2.Universitätshautklinik Wien
4.	T. mentagrophytes	Universitätshautklinik Genf
5.	T. mentagrophytes	CBS 56 066
6.	T. mentagrophytes var. quinckeanum	Sandoz Basel, No. 3667
7.	T. mentagrophytes var. quinckeanum	Institut für Mikrobiologie Bayer AG
8.	E. floccosum	2.Universitätshautklinik Wien
9.	E. floccosum	ATCC 15693
10.	M. canis	2.Universitätshautklinik Wien
11.	M. canis	ATCC 11622
12.	M. gypseum	2.Universitätshautklinik Wien
13.	M. racemosum	Hygiene Inst. Würzburg Prof. H. Seeliger, No.16
14.	Aspergillus fumigatus:	Sandoz Basel, No. 3609
15.	Sporotrichius schenkii:	ATCC 14804
16.	Candida albicans:	Sandoz Basel, No. 2869
17.	Candida parapsilosis:	Veterinärmed. Univ. Wien

2. Test Results

Test Strain	Minimum Inhibition Concentration µg/ml			
	Compound A	Compound B	Compound C	Compound D
1	≤0.1	100	100	100
2	≤0.1	100	100	100
3	≤0.1	>100	100	100
4	≤0.1	>100	100	100
5	≤0.1	100	100	50
6	≤0.1	100	100	100
7	≤0.1	100	100	100
8	≤0.1	100	100	100
9	≤0.1	>100	100	100
10	≤0.2	100	100	100
11	≤0.1	100	50	50
12	≤0.1	>100	100	100
13	≤0.1	>100	100	100
14	12.5	>100	100	>100
15	1.56	100	100	100
16	>100	>100	100	>100
17	3.13	>100	100	>100

Conclusions

In the in vitro tests set out above against various strains of dermatophytes, compound A is at least 500 times, and usually at least 1000 times, more active than any of compounds B, C and D. Indeed, in comparison with compound A, compounds B, C and D can be classed as inactive in these tests.

Furthermore, while the level of activity of compound A against the various other fungal strains tested is not so high as against dermatophytes, its activity against 3 out of of 4 of these strains is still vastly superior to that of compounds B, C and D.

Overall, in these tests, compound A is a highly active anti-mycotic agent, while compounds B, C and D are virtually inactive. Furthermore, this vastly differing spectrum of activity can in no way be explained by the fact that compound A was tested in hydrochloride salt form since the free base form would be expected to have even lower MIC values than the hydrochloride salt form, in view of the higher molecular weight of the latter.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issuing thereon.

this 16th day of August, 1979

Declarant _____

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